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14. ABSTRACT

Technologies such as transcriptional profiling, genome sequencing, SNP mapping, epigenetic, proteomic or metabolomic profiling have resulted in large sets of genome wide data. However, most of these datasets are of correlative. Functional and phenotypic annotation of the genome is thus a key challenge for a fundamental understanding of physiology and disease pathogenesis. We combine fly genetics with haploid ES cell mutagenesis and *in vivo* mouse genetics to functionally characterize candidate breast cancer genes. Using mouse genetics, we have now shown that inactivation of RANK markedly delays and in some cases even prevents the development of BRCA1-mutant breast cancer, providing a rationale for cancer prevention trials in BRCA1 carriers using RANKL blockade. We also performed a near-genome wide screen in Drosophila for genes that control tumor progression, allowing us to identify multiple novel tumor suppressor genes. Finally, we have now generated more than 100000 murine haploid ES cell clones targeting ~ 16500 different genes. We also developed new methods to generate blood vessels from haploid ES cells which allows us to genetically explore novel factors for tumor angiogenesis. One of these factors, Apelin, indeed cooperates with VEGFR signaling and, in mutant mouse models, appears to be a key regulator of tumor neoangiogenesis and survival in breast cancer.

15. SUBJECT TERMS

Genome wide functional genetics, haploid stem cells, *Drosophila* cancer modeling, breast cancer prevention, BRCA1 carriers

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INTRODUCTION:

With the advent of modern genomics hundreds of candidate genes have been associated with breast cancer both in GWAS studies as well as by cancer genome sequencing approaches. The big challenge is to determine which of these genes encode fundamental molecular pathways that initiate breast cancer and control metastases. It is also paramount to rapidly translate such findings into strategies for the prevention, future treatment, and possible eradication of breast cancer. My group generated the first RANKL mutant mice, providing definitive proof that RANKL is the master regulator of bone loss, opening a rationale avenue for novel therapies against bone loss in osteoporosis, leukemia, arthritis, or cancer metastases. We found another essential function for RANKL and its receptor RANK: formation of a lactating mammary gland in pregnancy. Moreover, we showed that RANKL/RANK function as soil factors for bone metastases of epithelial tumors, a finding that provided a mechanistic underpinning for clinical trials to delay bone metastases in prostate and breast cancer. Importantly, we also showed that the RANKL/RANK system is an essential molecular link between sex hormones and the development of breast cancer. In addition to environmental factors, genetic predispositions such as BRCA1 and BRCA2 mutation can cause high incidence and risk of developing breast cancer.

To capitalize on the breakthroughs in human genetics and genome engineering, in my grant I proposed to take the next step into the post-genome era of cancer, i.e. to develop systems to rapidly assess the function of candidate genes in breast cancer pathogenesis and metastases. To uncover and confirm novel genes with direct relevance to breast cancer, I proposed a multifaceted approach to combine the power of *Drosophila* genetics with our novel haploid ES cell technologies and finally *in vivo* cancer modelling using mouse genetics. Moreover, we continue to work on the role of RANKL/RANK in mammary cancer. Validation of RANKL/RANK as critical molecules in breast cancer initiation, mammary stem cell biology, and metastasis and the recent generation of therapeutics directed to block RANKL will have a direct impact for breast cancer patients.

BODY:

The following three tasks were proposed

1. **Dissecting the role of RANKL/RANK in mammary cancer.**
2. **Genetic approaches to identify novel cancer genes and tumor suppressors.**
3. **Modelling cancer pathways using haploid stem cell technologies.**

1. Dissecting the role of RANKL/RANK in mammary cancer.

Background: The research builds on our previous work where we provided the first genetic evidence that a molecule called RANKL is essential for bone turnover via osteoclasts in healthy individuals and bone loss in millions of patients suffering from osteoporosis or rheumatoid arthritis[1,2]. We then found that RANKL and its receptor RANK controls the proliferation of mammary epithelial cells essential for lactating cells during pregnancy[3], a finding that provided a molecular and evolutionary explanation for gender bias and the high incidence of osteoporosis in females. Based on the clinical importance of RANKL inhibition already being approved for bone loss in osteoporosis and skeletal related event in cancer[4,5] and the essential physiological function of RANKL/RANK in mammary epithelial proliferation in pregnancy in response to sex hormones[3,6], we initially came up with the idea that RANKL/RANK might play a role in breast cancer initiation. My group[7] and Gonzalez-Suarez *et al*.[8] were indeed able to show that the RANKL-RANK system is a key regulator of hormone (progestin)- and oncogene (Neu)-driven mammary cancer. Intriguingly, therapeutic inhibition of RANKL can reduce the incidence of hormone driven breast cancer from 100 to ~ 10% in a mouse model.

We have also shown that RANKL and RANK are expressed in primary breast cancers and breast cancer-derived cell lines and that the RANKL/RANK system can function as a soil factor that controls bone metastases[9], a finding that provided, among work from other groups[10,11], a rationale for clinical trials to delay bone metastases in prostate and breast cancer. In prostate cancer, blocking RANKL can indeed delay the first occurrence of bone metastases[12]. The already

FDA approved RANKL blocking antibody (Denosumab) is currently being tested in phase III clinical trials preventing metastases in breast cancer patients. Moreover, RANKL inhibition is now being tested in a phase III clinical trial in an adjuvant therapeutic setting and recently it has been reported that RANKL should be considered a standard therapy to prevent broken bones in breast patients that receive aromatase inhibitor treatment. Thus, after we proposed that RANKL/RANK might have a role in breast cancer[3], RANKL inhibition has already become therapy for thousands of women with breast cancer and, based on the outcome of the ongoing phase III trials, has the promise to even prevent metastases and delay the development of a second tumor in adjuvant therapy.

Results: One key question is whether RANKL inhibition could be one day used for prevention of breast cancer. To accomplish this, we however need to provide direct evidence that inhibition of RANKL and its receptor RANK can indeed delay the occurrence of mammary cancer in high risk mammary cancer scenarios. Risk factors for breast cancer development include exposure to environmental factors such as synthetic sex steroid hormones[13,14], or genetic predisposition. In particular, germline mutations in *BRCA1* and *BRCA2* account for 2-10% of breast cancer cases depending on the ethnic population[15]. Tumors from *BRCA1* mutation carriers are in general triple negative "basal" phenotype and thus have a bad prognosis and limited treatment options. Although BRCA1/2 are involved in the repair of double strand breaks in DNA, human evidence suggests a relationship between *BRCA1/2* mutations, sex hormone levels, and cancer risk[16]. In addition, progesterone has been shown to play a role in mammary tumorigenesis of *Brca1/p53* double mutant deficient mice[17]. We therefore focused our efforts to investigate the role of RANKL/RANK in *BRCA1*-mutant breast cancer.

To directly assess the role of RANKL/RANK in *Brca1* mutation-mediated tumorigenesis, we deleted *Brca1* plus *p53* in basal mammary epithelial cells and mammary progenitor cells using K5Cre mice to induce spontaneous mammary cancer as previously reported[18,19.] This line was then crossed into $Rank^{flox/flox}$ mice to examine spontaneous tumorigenesis in the presence or absence of RANK expression. Importantly, at 4 month of age we observed spontaneous and widespread epithelial hyperplasia (Figure 1a) as well as low and high grade mammary

intraepithelial neoplasias (MIN) and also invasive carcinomas in *K5Cre;Brca1;p53* double mutant mice (n=8) (Figure 1b). The mammary glands of age-matched females with concomitant ablation of *Rank* (n=8) appeared largely normal displaying a significantly lower number of low grade and high grade MINs and we never detected any carcinomas (Figure 1b). We observed marked DNA damage in both double and triple mutant mice as determined by γH2Ax immunostaining. These data show that genetic deletion of *Rank* in basal mammary epithelial cells markedly abrogates the development of intraepithelial neoplasias and invasive carcinomas as a consequence of *Brca1;p53* mutations.

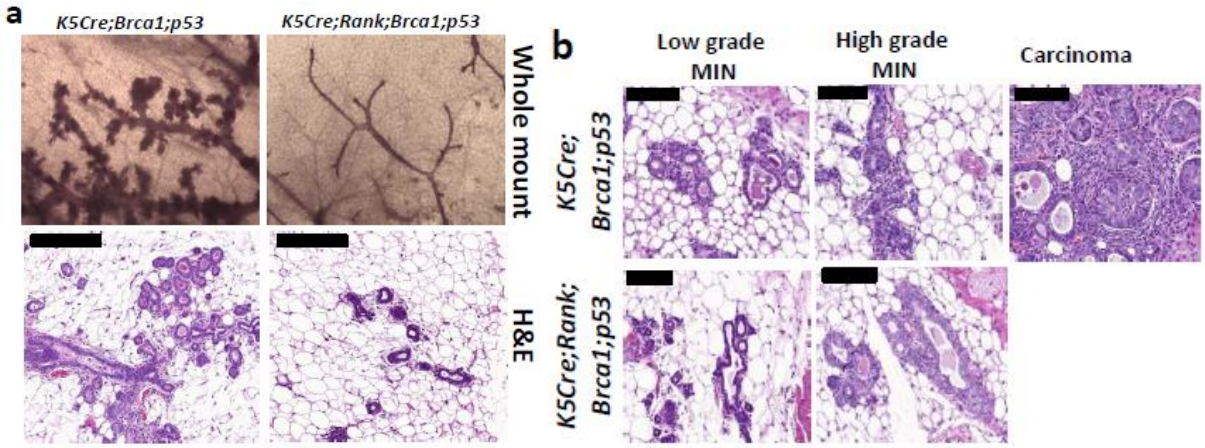

Figure 1. Ablation of *Rank* in basal mammary epithelial cells markedly decreases tumor formation in *Brca1/p53* mutant female mice. a, Representative whole mount images (haematoxylin staining, magnifications 52x) and paraffin sections (H&E staining, scale bar represents 200µm) of mammary glands from 4 month old *K5Cre;Brca1;p53* double and *K5Cre;Rank;Brca1;p53* triple knockout littermate mice. **b,** Representative images (H&E staining, scale bars represent 100µm) of low grade MINs, high grade MINs and adenocarcinomas in mammary glands from 4 month old *K5Cre;Brca1;p53* and *K5Cre;Rank;Brca1;p53* mutant littermates.

The occurrence of skin cancer commonly observed in the *K5Cre;Brca1;p53* double[18,19] as well as *K5Cre;Rank;Brca1;p53* triple mutant mice precluded further analysis of mammary tumorigenesis beyond the 4 month time-point. We therefore switched the Cre deleter line and introduced all three conditional alleles onto a WapCre^{C} mouse background[17]. Of note, in this mouse line, the Whey-acid protein (Wap)rTACre activity is independent of doxycyclin and pregnancy and the

Cre activity present in both luminal and basal epithelial cells in the mammary gland**.** WapCre^{C}-mediated *Rank* deletion had no apparent effect on formation of the mammary gland during puberty. While all *WapCre^{C};Brca1;p53* mutant females developed spontaneous palpable tumors starting around day 100 after birth, concomitant *Rank* deletion in the mammary epithelium resulted in a delayed tumor onset. The median day of onset for *WapCre^{C};Brca1;p53* mice was 158 days, whereas the median day of onset for *WapCre^{C};Rank;Brca1;p53* triple mutant mice was 184 days. Importantly, while all *WapCre^{C};Brca1;p53* double mutant females developed spontaneous mammary tumors, 25% of *WapCre^{C};Rank;Brca1;p53* triple mutant littermates never developed any tumors which was also reflected by the overall survival rates (Figure 2, left panel), even when we followed these females throughout their lifetimes, sometimes up to 2 years of age. Once mammary tumors developed in both double and triple mutant mice, their growth curves appeared to be similar. Thus, *Rank* inactivation using WapCre^{C}-driven deletion delays the onset and in some cases even completely prevents the development of *Brca1;p53* mutation-driven spontaneous mammary cancer.

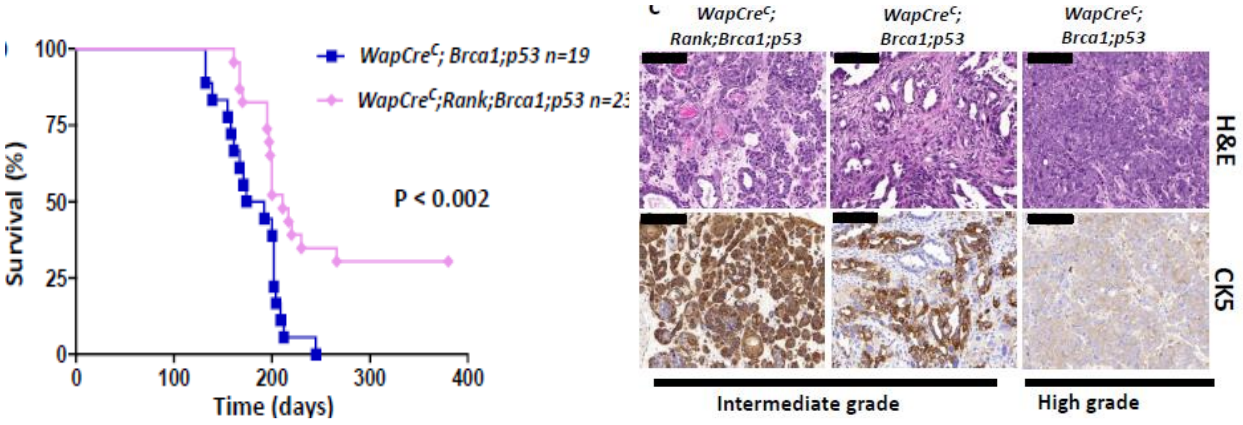

Figure 2. Figure 2. *Rank* deletion reduces the incidence of *Brca1* and *p53* mutation-driven mammary tumorigenesis. Left panel, Overall survival rates in *WapCre^{C};Brca1;p53* and *WapCre^{C};Rank;Brca1;p53* mice. Data are shown as percentage of overall mouse survival. **Right panel,** Representative histological sections of mammary tumors isolated from *WapCre^{C};Brca1;p53* and *WapCre^{C};Rank;Brca1;p53* mice showing different histological grades (H&E) and altered, grade-dependent Cytokeratin 5 immunostainings (CK5). Scale bars represent 100µm.

Histopathologic analysis showed that mammary tumors from both genotypes exhibit typical signs of intermediate grade cancers; however high grade cancers were only observed in mammary tumors from *WapCre^{C};Brca1;p53* double mutant females (Figure 2, right panel). All intermediate grade tumors that developed in double and triple mutant mice expressed Cytokeratin5^{+} (CK5) indicative of a basal cell subtype. However, in high grade tumors from *WapCre^{C};Brca1;p53* double mutant females CK5 expression was in most cases lost, further supporting the notion of marked epithelial dedifferentiation of such mammary cancers (Figure 2, right panel). CK5 positive low to intermediate grade foci and K5 negative high grade foci were often found within the same tumor. We failed to detect metastases into the lung in either double or triple mutant mice that had developed tumors. Of note, whereas RANK was expressed in all mammary tumors analysed in *WapCre^{C};Brca1;p53* females, RANK expression was absent in tumors from *WapCre^{C};Rank;Brca1;p53* triple mutant females as determined by immunohistochemistry or qPCR. Thus, loss of RANK not only delays tumor onset, but also affects progression to high tumor malignancy.

Ki67 immunostaining of mammary tumors from *WapCre^{C};Brca1;p53* double mutant and *WapCre^{C};Rank;Brca1;p53* triple mutant females showed comparable proliferation of tumor cells in both genotypes, a finding that might explain that established tumor grow at a comparable "speed". Finally, immunostaining for γH2Ax showed that loss of Brca1 and p53 resulted in massive DNA damage in intermediate as well as high grade tumors, irrespective of the presence or absence of RANK expression). Importantly, we also observed marked DNA damage in the mammary epithelium of a two year old *WapCre^{C};Rank;Brca1;p53* triple mutant female that had never developed any tumor (Figure 3). Although there was substantial DNA damage, the mammary glands appeared histologically normal and only very few mammary epithelial cells proliferated as determined by very low Ki67 positivity. Analysis of mammary epithelial cells (MECs) isolated from the tumor-free 2 year old *WapCre;Rank;Brca1;p53* female confirmed efficient deletion of *Brca1*, *p53* as well as *Rank*. These data indicate that loss of *Rank* protected from mammary tumorigenesis despite the presence of DNA damage due to the inactivation of *Brca1*.

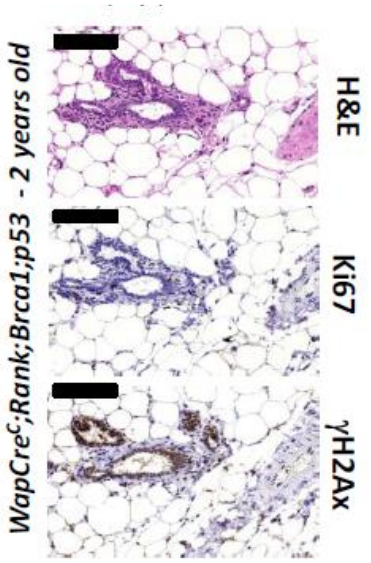

Figure 3. *Rank* deletion can prevent from *Brca1* and *p53* mutation-driven mammary tumorigenesis. H&E staining, Ki67 and γH2Ax immunostainings of mammary glands from a 2 year old tumor free WapCre^{C};Rank;Brca1;p53 mutant mouse, showing apparently normal mammary gland tissue, low Ki67 positivity and DNA damage indicated by γH2Ax positivity**.** Scale bars represent 100µm.

Since RANK controls key mitogenic pathways that may influence stem/progenitor cells and its deletion delays onset and in some cases even prevents *Brca1;p53*-mutation driven mammary tumors using two different Cre deleter mouse lines, we speculated that RANKL/RANK might affect proliferation and expansion of mammary progenitor cells on the *Brca1;p53* mutant background. To assess if RANKL/RANK control expansion of *Brca1;p53* mutant mammary progenitor cells, we performed ovariectomy (ovx) on females of all genotypes followed by sham treatment (no hormones) or reconstitution with estrogen and progesterone (E+P) to control for sex hormone levels. As previously reported[20,21], sex hormone treatment resulted in a marked expansion of Lin^{-}CD24^{+}CD49f^{hi} basal mammary progenitor cells in both control as well as *WapCre^{C};Brca1;p53* double mutant mice; however, this expansion was markedly reduced in the absence of RANK expression (Figure 4a,b). Sex hormone-driven proliferation in ovx females was further determined by *in vivo* BrdU labelling. WapCre^{C}-driven deletion of *Brca1* and *p53* resulted in markedly increased numbers of cycling mammary epithelial cells; this sex hormone-induced proliferation of mammary epithelial cells was significantly reduced in *Rank* mutant females). Importantly, deletion of *Rank* also markedly abrogated the *in vitro* colony formation capacity of

basal Lin$^-$CD24$^+$CD49f^{hi} mammary progenitors from *WapCre^C;Brca1;p53* mutant mice; the progenitor capacity within the ER$^-$PR$^-$ alveolar progenitor Sca1$^-$ compartment was also lower in the absence of RANK). Similar to our data using WapCre^C deleter mice, sex hormone treatment resulted in expansion of the basal Lin$^-$CD24$^+$CD49f^{hi} progenitor compartment in ovarectomized *K5Cre;Brca1;p53* double knockout mice as compared to sham operated mice, while this expansion was not observed in the absence of RANK. Thus, RANKL/RANK controls sex-hormone induced expansion and activity of *Brca1;p53* mutant basal progenitor cells.

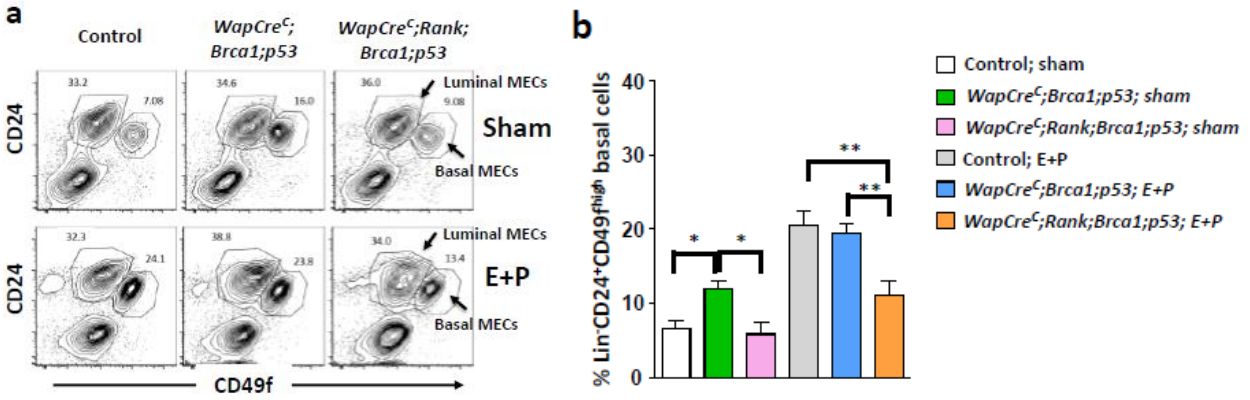

Figure 4. RANK mediates proliferation and expansion of *Brca1;p53* mutant basal mammary epithelial cells in response to hormonal stimulation. a, Representative FACS profiles of luminal (CD24$^+$CD49f^{lo}) and basal (CD24$^+$CD49f^{hi}) mammary epithelial cell populations (MECs) in ovariectomized WapCre^C-negative control, *WapCre^C;Brca1;p53* double knockout and *WapCre^C;Rank:Brca1;p53* triple knockout female mice treated with 17β-estradiol plus progesterone (E+P) or left untreated (sham). **b,** Quantification of CD24$^+$CD49f^{hi} basal mammary epithelial cells in ovariectomized WapCre-negative control, *WapCre^C;Brca1;p53* double and *WapCre^C;Rank:Brca1;p53* triple knockout mice treated with E+P or without hormone treatment (sham). Data are from 10-12 old mice, at which age we never observed any evident tumors, and represent mean +/- SEM of at least n=3 mice/group. * $p<0.05$; **$p<0.005$; Student's t-Test.

Our results show that genetic inactivation of RANK has a marked effect on the onset of mammary tumors and also survival of female mice that carry *Brca1;p53* mutations. It has been recently shown that RANK expression is markedly increased in triple negative breast tumors from *BRCA1* carriers compared with other subtypes[22]. RANKL acts in a paracrine fashion on the

membranous RANK of ER/PR-negative epithelial cells of the breast to expand the stem and progenitor cell populations[20,21]. Our data show that RANKL/RANK also regulates the expansion and functional capacity of progenitor cells on a *Brca1/p53* mutant background. Since RANKL is induced by progesterone, these data could explain why sex hormones, in particular progesterone, play an important role in *BRCA1* mutation-driven tumorigenesis in humans and mouse models. Future mechanistic studies are required to test the hypothesis that the abnormal, sustained accumulation of hormone receptor negative cells in *Brca1* deficient mammary glands is due, in part, to a dysregulated RANKL/RANK-driven expansion of progenitor cells.

Taken together these data show that RANKL/RANK link sex hormones to progenitor cell expansion and tumorigenesis in inherited breast cancer and provide a viable strategy to possibly prevent breast cancer in patients with *Brca1* mutations. These results also provide a rationale to initiate phase II/III breast cancer prevention trials in humans BRCA1 carriers using the already FDA-approved anti-RANKL antibody. First discussion with clinicians and industry sponsors of such clinical prevention trials have been initiated.

2. Genetic approaches to identify novel cancer genes and tumor suppressors.

Background: Epithelial tumors are a major cause of morbidity and death in the world[23]. In many cases cancer death occurs via spreading of the primary tumor to secondary sites forming metastases. Cancer metastasis is a complex process including altered cell adhesion, invasion, intravasation and extravasation and secondary site tumor growth, allowing for a physical relocation of primary tumor cells to secondary organs. Considering millions of cancer patients, in particular in an aging population, it is therefore paramount to identify novel pathways and genes involved in cancer growth and metastatic dissemination.

Many developmental pathways and signaling cascades implicated in cancer have been originally identified in *Drosophila melanogaster*[24-26] and oncogenic signaling pathways such as Ras and Notch are conserved from fly to human[27]. Moreover, epithelial tumor cell metastasis has been

modeled in fly larvae[28,29]. Thus, fundamental mechanisms of oncogenic transformation and metastases are highly conserved among flies and humans. Researchers at our campus have created the largest global RNA interference library in the world representative of nearly all expressed *Drosophila* genes; thus we have local access to a library of *Drosophila* where every fly gene can be inactivated via RNAi *in vivo* using tissue specific promoters[30]. We used this library to perform a near genome-wide screen to identify genes involved in the growth, invasion, and metastasis of epithelial cells.

Results: In an attempt to identify genes involved in cancer development and metastases, we designed a high through-put *in vivo* RNAi screening system for genes involved in progression of oncogenic Ras-driven epithelial tumors. An activating point mutation in codon 12 of Ras, Ras^{V12}, is one of the most frequent mutations found in human patients. Using our experimental set-up we performed a large scale *Drosophila* screen to identify novel genes that modulate $dRas^{V12}$-induced epithelial tumorigenesis at a systems level. Female *Drosophila* carrying the $dRas^{V12}$ oncogene were crossed to male flies containing *UAS-RNAi* transgenes resulting in progeny that expresses $dRas^{V12}$ and the respective RNAi transgenes specifically in the developing eye-antennal disc epithelium. We tested 10,720 *UAS-RNAi* lines corresponding to evolutionary conserved 6675 genes, which allowed us to identify hundreds of novel genes that modulate Ras-induced transformation. Expression of the human orthologs of our primary fly hits were significantly ($p < 0.00002$) downregulated in thousands of human tumors making fly a viable model system to study human cancer. Based on our human gene expression profiles, we were able to construct a heatmap of the top hundred genes with the lowest expression scores in different human cancers as compared to their respective normal tissue controls (Figure 5). The fly orthologs of these top one hundred candidate human genes were then retested in a secondary screen in *Drosophila* using the *ey-GAL4 UAS-dRasV12* system. In this system, expression of oncogenic $dRas^{V12}$ in the eye epithelium results in a rough adult eye phenotype due to epithelial hyperplasia that can be then scored for *dRasV12* cooperating genes. Together with *dRasV12* overexpression, 80% of genes retested in this setting showed increased hyperplasia of the eye epithelium (Figure 6). These results collectively validate the top 100 candidate *dRasV12* cooperative tumor suppressors

and point towards evolutionary conservation of genes that control epithelial tumor progression. Importantly, we also identified multiple novel candidate tumor suppressors.

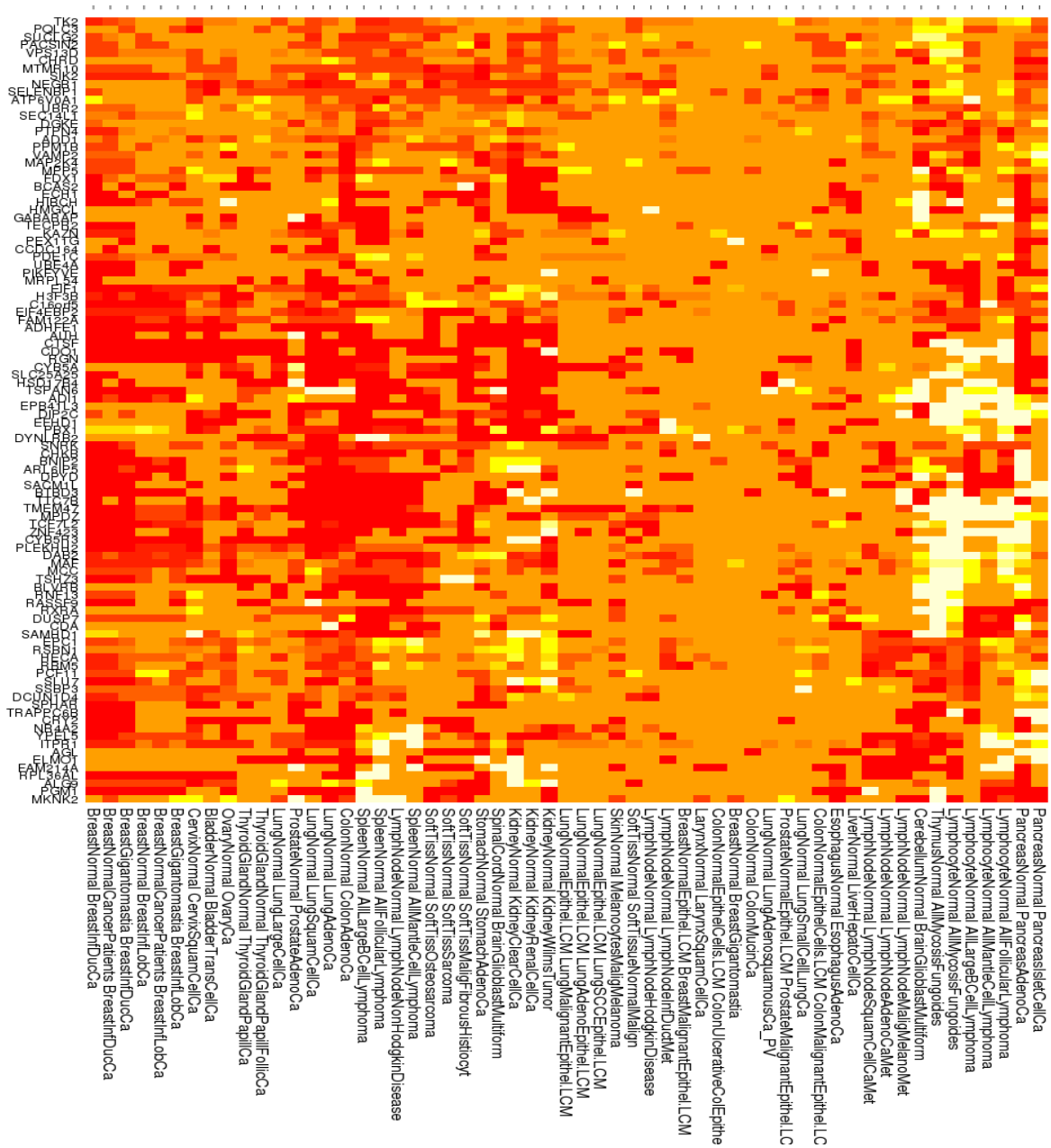

Figure 5. Heatmap for the top 100 human ortholog candidate tumor suppressor genes tested for differential expression (log2 values equivalent to fold change) between a cancer sample set and its corresponding normal tissue. The gene symbol and the Affymetrix probe set name are listed. Differential expression between tumor and the corresponding normal tissue is color coded as follows: red-lower expression in tumors, orange -no significant difference, white and yellow-increased expression in tumors.

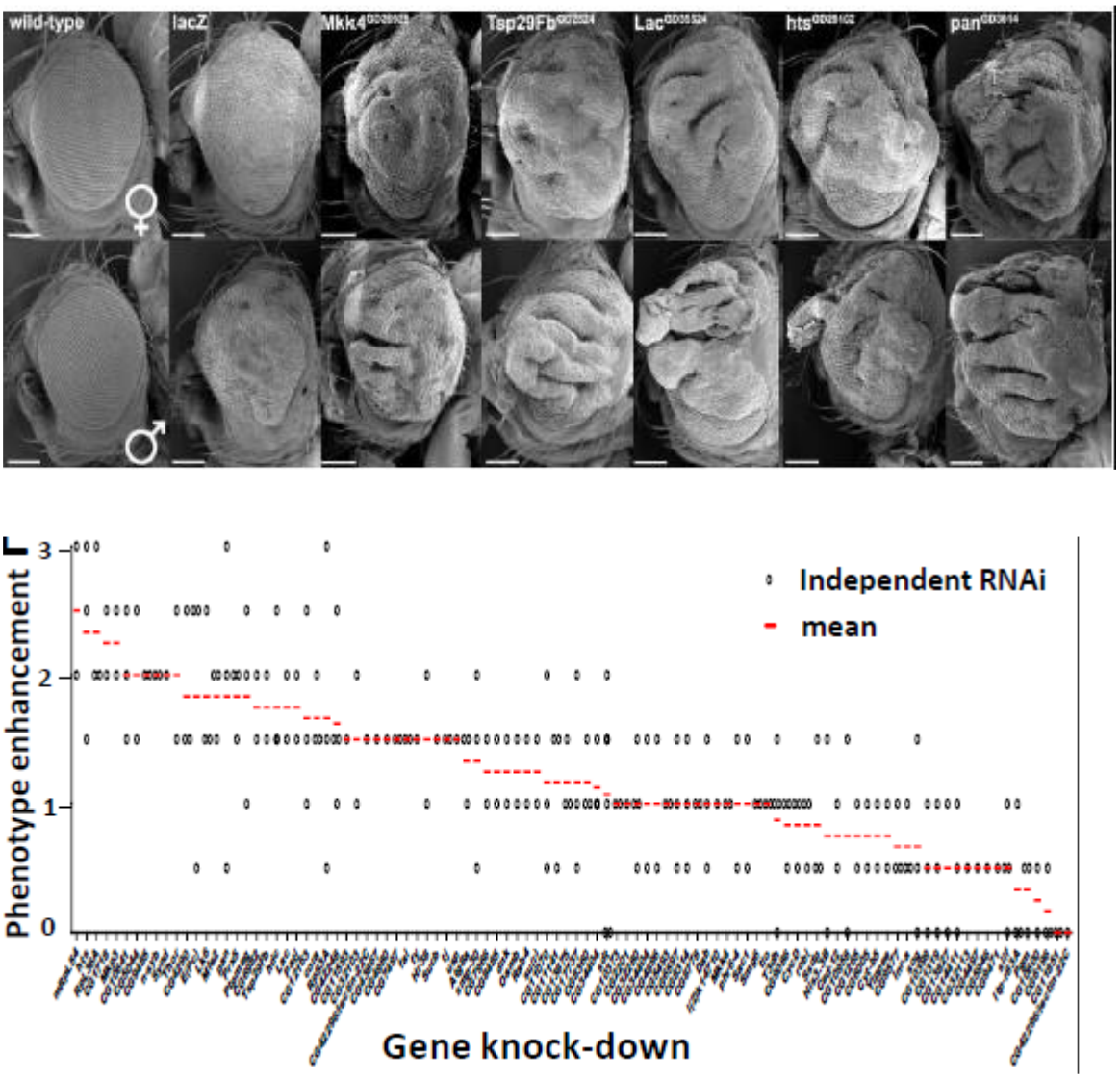

Figure 6. Validation screens in flies. Top panel, Representative scanning electron micrographs of *dRasV12*-cooperative eye overgrowth shown for both females and males in a secondary RNAi screen for 100 genes with the best differential expression score in human tumors. Genotypes from top left: wild-type, *ey>dRasV12/lacZ, ey>dRasV12/mkk4-RNAi, ey>dRasV12/Tsp29Fb-RNAi, ey> dRasV12/Lac--RNAi, ey> dRasV12 /hts--RNAi, ey> dRasV12/pan--RNAi* (scale bar = 100μm). **Bottom panel,** Enhancement of *dRasV12*-driven eye overgrowth by RNAi lines representing the top 100 genes down regulated in human tumours. 0 = no enhancement, 1 = enhancement, 2 = strong enhancement, 3 = very strong enhancement. The diamond indicates scores for individual RNAi lines tested. The red line represents the mean of phenotype scores when gene knockdown effect was tested with multiple RNAi lines.

25 of our validated hits could not be assigned to known signaling pathways and genetic networks, suggesting that these may be novel, functionally yet uncharacterized cancer genes. Among these 25 candidates, the strongest phenotypes in the secondary screen were observed for the three following *Drosophila* genes: *CG1064*, *smp-30* and *Tsp29Fb* corresponding to the human genes *EFHD1*, *RGN*, and *TSPAN6*, respectively. When comparing invasive phenotype scores observed in our primary screen assay, downregulation of *EFHD1* and *Tsp29Fb* revealed the strongest cooperation with $dRas^{V12}$ as evidenced by secondary foci formation and dissemination of single GFP+ tumor cells. Among these three candidates, *EFHD1* and *RGN* have been previously implicated to have tumor suppressor functions. Functional involvement of Tsp29Fb/TSPAN6, a member of the tetraspanin family coding for transmembrane-spanning proteins, in tumor growth and progression has not been reported thus far. In humans and mice, *TSPAN6* mRNA is expressed in multiple epithelial tissues including the mammary gland**.** Therefore, we focused on the functional characterization of fly Tsp29Fb and its mammalian ortholog TSPAN6.

To explore the role of TSPAN6 in mammalian cancer, we generated *TSPAN6* whole body knock-out mice as well as $TSPAN6^{floxed}$ mice using homologous recombination. These mice are viable and have no overt macroscopic phenotype. Our functional data show that TSPAN6 restrains growth and metastasis of human and murine Ras-mutant epithelial cancer cells and clonal inactivation of TSPAN6 in mice markedly enhanced $Kras^{G12D}$-driven initiation and malignant cancer progression. Mechanistically, both fly Tsp29Fb and mammalian TSPAN6 control Ras and EGFR activation and affect morphology, migration, and fate of epithelial cells. The whole-body and epithelial-specific TSPAN*6* mutant females were also challenged with MPA and DMBA to induce hormone-driven mammary tumors. Importantly, we observed markedly enhanced onset of first palpable tumors and reduced overall survival of the mice (Figure 7). Similar to the whole body mutant mice, deletion of TSPAN6 in the mammary epithelium markedly enhanced mammary tumor onset and significantly reduced the survival of the female mice (Figure 8). Our evolutionary approach to identify novel players in cancer progression allowed us to identify a novel gene, TSPAN6, that affects epithelial architecture and restrains tumor growth and metastasis.

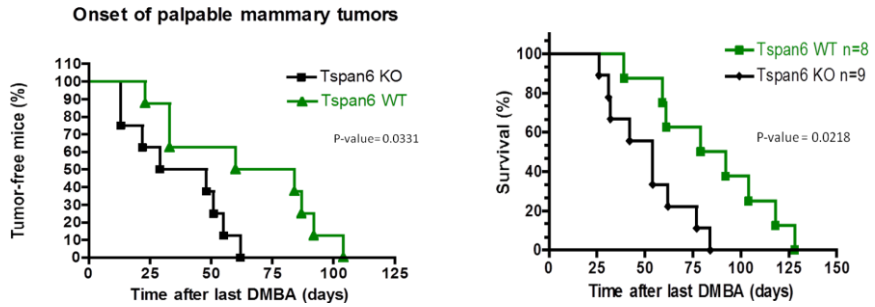

Figure 7. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in TSPAN6 expressing and TSPAN6 mutant age-matched littermate control females treated with the progestin MPA and the carcinogen DMBA. For MPA/DMBA carcinogenesis, nulliparous, six-week old female mice were s.c. implanted with MPA pellets and treated orally with DMBA as indicated for 8 weeks. Data are shown as percentage of tumor free mice after the last DMBA challenge.

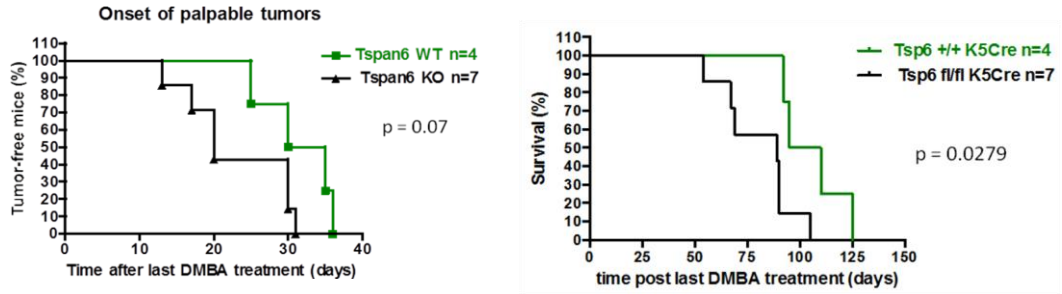

Figure 8. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in K5-Cre TSPAN6fl/fl and TSPAN6 expressing control littermates treated with the progestin MPA and the carcinogen DMBA. Data are shown as percentage of tumor free mice after the last DMBA challenge.

SNP chip analysis of human tumor cell lines revealed that the *TSPAN6* locus in the Xq22.1 genomic region is frequently deleted in breast cancer cells: 24/59 cell lines analyzed showed chromosomal deletions encompassing the *TSPAN6* locus. We also observed deletions spanning the TSPAN6 locus in primary breast cancer. Importantly, typical for tumor suppressors, we detected defined focal deletions and break-points at the genomic region containing the *TSPAN6* locus in primary human breast tumors. Thus, TSPAN6 is commonly down-regulated in multiple human breast cancers and the genomic region encompassing the *TSPAN6* locus is frequently deleted in breast tumor cell lines and primary human breast cancer. Since the biological

mechanisms of tumor development and progression in breast cancer differ according to their molecular phenotype[31-34], we evaluated TSPAN6 mRNA expression in three major molecular subtypes of breast cancer, i.e. ER-HER2- ("basal-like"), ER-HER2+ ("HER2-overexpressing"), and ER+HER2- ("luminal") subtypes. Intriguingly, whereas TSPAN6 mRNA levels were not prognostic in patients with ER-HER2- and ER-HER2+ tumors (not shown), we observed a significant association of TSPAN6 expression with metastases-free survival in patients diagnosed with ER+HER2- breast cancer. Among patients with ER+ breast tumors, TSPAN6low mRNA expression is associated with advanced tumor grades, poorer survival, and enhanced risk of distant metastases. TSPAN6low expression also predicts poor clinical outcome even in luminal-A patients with lymph node negative disease. Thus, TSPAN6 appears to be a novel prognostic biomarker for ER+ breast cancer and TSPAN6high expression defines a subpopulation of ER+ breast cancer patients with very good clinical prognosis. Thus, TSPAN6 appears to function as a novel tumor suppressor for breast cancer.

3. Modelling cancer pathways using haploid stem cell technologies.

Background: Genetic screens have markedly contributed to the understanding of basic physiology and disease pathways[35]. A drawback of genetic screens in cell culture is that one needs to derive cells from homozygously mutated animals or target a gene twice since mammalian cell culture systems are unable to undergo sexual reproduction cycles[36]. Some organisms such as yeast or males of social insects are haploid, i.e. they carry a single set of chromosomes, while haplopidy in mammals is exclusively restricted to mature germ cells. A single copy of the genome provides the basis for genetic analyses where any recessive mutation of essential genes will show a clear phenotype due to the absence of a second gene copy. Most prominently, haploidy in yeast, has been utilized for recessive genetic screens that markedly contributed to our understanding of normal development, basic physiology, and disease. Somatic mammalian cells carry two copies of chromosomes (diploidy) that obscure genetic analysis. Near haploid leukemic cells however have recently been developed as a high throughput screening tool[37]. Although deemed impossible, we generated mammalian haploid embryonic stem cells

from parthenogenetic mouse embryos, thereby opening haploid screening technology to a wide range of biological questions[38]. Haploid stem cells open the possibility of combining the power of a haploid genome with pluripotency of embryonic stem cells to uncover fundamental biological processes in defined cell types at a genomic scale. Haploid genetics has thus become a promising powerful and rapid alternative to RNAi or CRISPR based screens.

Results: My group was among the first in the world to develop haploid ES cells, i.e. stem cells with a single set of chromosomes[38]. We now spent considerable efforts to develop very efficient gene trap vectors for genome-wide mutagenesis that are conditional and can be reverted to wild type via Cre-recombinase as well as back to a null allele via Flippase thereby allowing immediate confirmation of the candidate gene[39]. After mutagenesis at the haploid stage, these cells duplicate the genome within 1-2 weeks to become diploid, i.e. we can evaluate the effects of mutations in a close to normal genetic setting. Moreover, each clone carries its own genetic barcode to be able to perform quantitative genome-wide screens. Since each system has defined hotspots of integration, we cloned and established multiple insertional mutagenesis vectors, based on retrovirus and lentivirus backbones as well as *Sleeping Beauty* and *Tol2* transposon. We now routinely generate between 50-700 Million integrations in a single experiment using about 1 billion sorted haploid ES cells as the starting point. Importantly, when combined we nearly hit every protein coding gene in the genome. Thus, we have been able to successfully set up a system that makes it possible to perform genome-wide, unbiased insertional mutagenesis. The resulting clones carry insertions that can be repaired and carry a barcode. The surviving cells can be selected, for instance selected for synthetic lethality testing cancer drugs on sensitized background (backgrounds we can either engineer or develop from mutant mouse strains such as BRCA1/p53 mutant mice), and the integrations mapped using 4D Sudoku sequencing. Thus, within a short time-period it is now possible to identify essential pathways implicated in e.g. the survival of our cells in response to cytotoxic cancer drugs. Figure 9 shows the insertional, screening, retrieval of mutations and screen analyses we have developed over the last years.

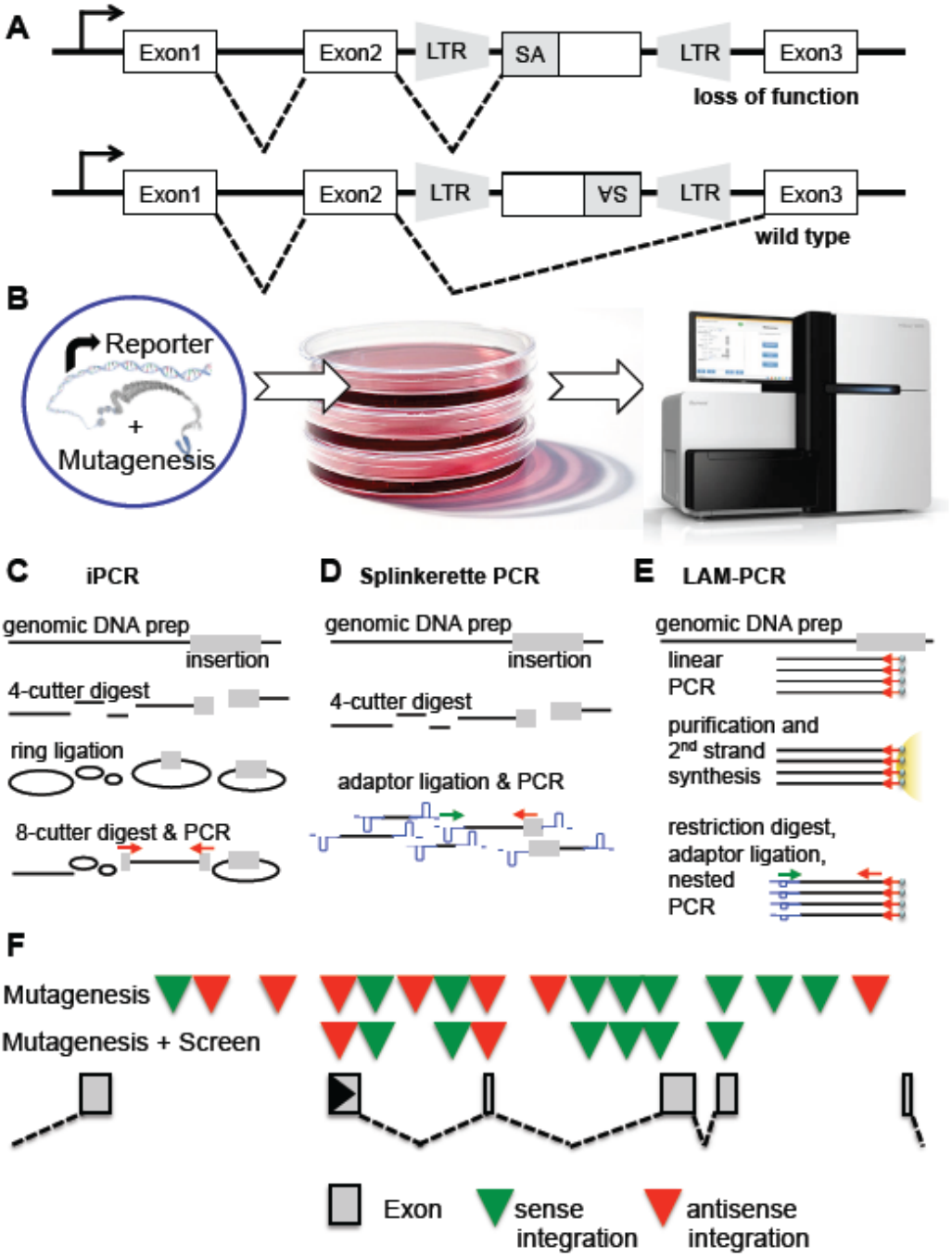

Figure 9. Insertional mutagenesis, screening, retrieval of mutations, and screen analysis. A) Insertional mutagens such as transposons or retroviruses (LTR: long terminal repeat) can be used to deliver splice acceptors (SA) into the genome. If splice acceptors are inserted in introns in sense relative to the transcripts, they will trap them and lead to premature termination and loss of gene expression. Antisense integration will leave gene transcripts unharmed in most cases. **B)** Diagram showing principles of haploid screens. Upon optional reporter integration and mutagenesis, cells are selected from a large pool. Thereby, millions of independent mutations can

be assayed using only a few plates within weeks. Extracted DNA is processed and subjected to deep sequencing using e.g. an illumina platform. Different PCR based methods allow for the identification of multiple integration sites in parallel. **C-E)** Inverse PCR relies on ring ligation of digested fragments and uses 2 locus specific primers (red), while splinkerette PCR ligates linkers (splinkerette) to restriction digest fragments and thus uses only 1 locus specific primer. Splinkerette structure allows for initial priming only from the specific primer. LAM-PCR (linear amplification mediated PCR) is based on linear PCR with tagged primers. Tags allow for bead based single stranded DNA purification followed by second strand synthesis and semi-nested PCR upon tag ligation similar to splinkerette PCR. **F)** Insertional mutagens will integrate in sense (green) or antisense (red) at inter- as well as intragenic regions. Upon selection, exonic as well as intronic sense integrations are expected to remain in loci with selected loss of function phenotypes.

Importantly, using our murine haploid embryonic stem cells und the above described mutagenesis system, we have initiated a cell bank, called Haplobank, in order to develop a library of ES cell clones where nearly every expressed gene carries a homozygous, repairable and bar-coded loss of function mutation. We initially proposed to derive, isolate, expand, freeze and map ~ 50.000 independent mutant clones as an initial step towards the goal of a genome wide collection of null alleles, not knowing about the frequencies of insertional mutagenesis, insertional hotspots etc. We have now picked and mapped more than 90000 individual ES cell clone. Our insertions are associated with more than 16500 different genes. 11312 ES cell clones with intragenic integrations in protein coding genes carry a single integration (Figure 10). Thus, we have set-up mutagenesis systems with various vector systems that will allow us to perform near-genome saturated forward genetics and we have generated ES cell clones that now can be used for reverse genetics and will be made available to all researchers. We have also started to clone a CRIPR-Cas9 based library to combine this system of genome engineering with the power of haploid genetics.

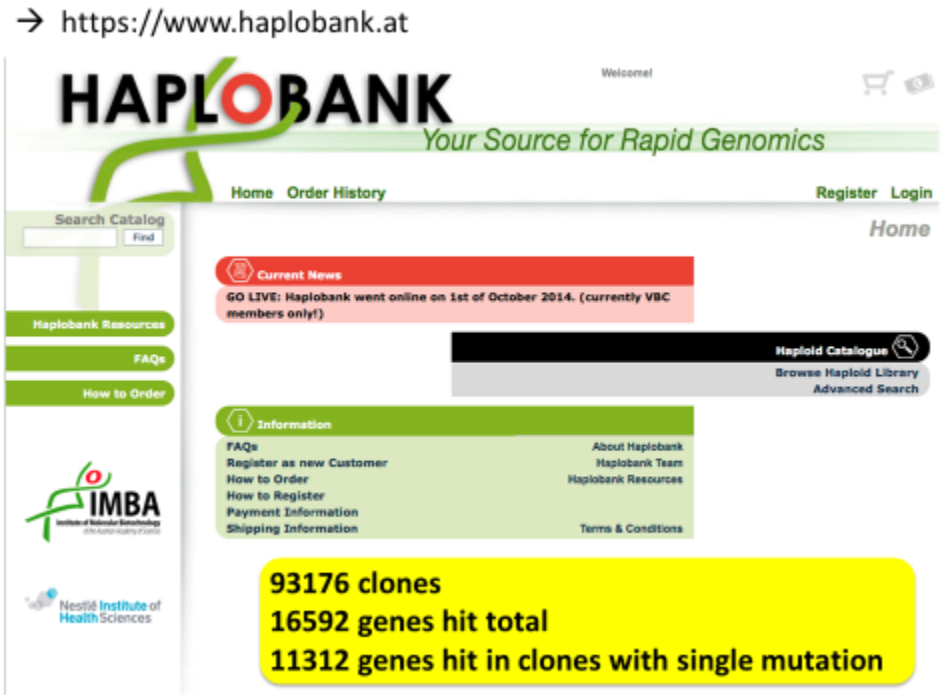

Figure 10. Numbers of total clones mapped, single and multiple insertions in individual mouse haploid ES cell clones and the numbers and frequencies of intragenic insertions. The bottom panel shows total numbers of genes carrying intragenic insertions, total genes hit, and clones with mapped single intragenic insertions. This library will be freely accessibly (for cost) to all academic researchers in the world to promote cancer research.

Using the power of this library we can now model, for instance, blood vessel development from our stem cells. We indeed managed to develop such model starting from haploid ES cells that can be differentiated into embryoid bodies and then further into 3D blood vessel organoids following addition of VEGF-A in collagen matrix (Figure 11). These organoids contain endothelial cells, pericytes, and a basal membrane, and form perfect lumens. Moreover, we can transplant the organoids and also the embryoid bodies to study blood vessel formation in vivo mouse models. Of note, since the mutations in the haploid ES cells are repairable, we can directly compare the function-side, thereby avoiding potential pitfalls of clonal effects in different cell lines. This tissue engineering approach now allows us to test the role of multiple known and predicted modifiers of angiogenesis, in particular to assess the functions of possible modulators of tumor angiogenesis.

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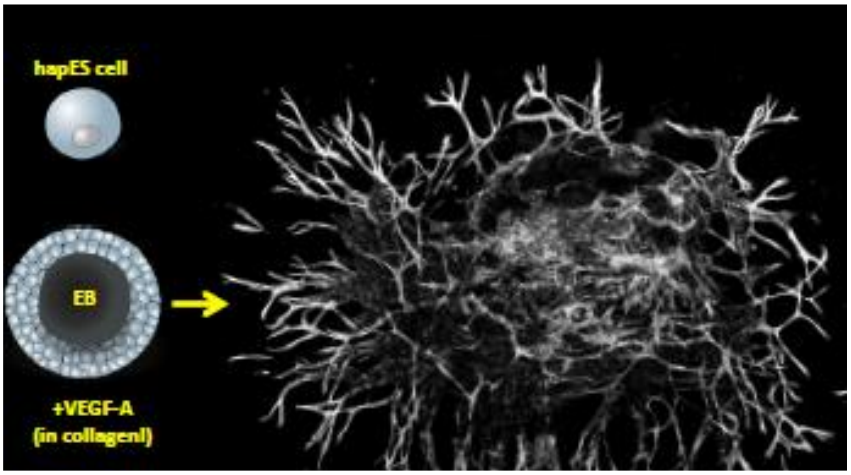

Figure 11. Schematic outline of blood vessel development from haploid ES cells (hapES cell) and embryo bodies (EB). The image shows a $CD31^{+}$ endothelial 3D organoid.

As tumor growth depends on the formation of a neovascular supply, inhibition of tumor-induced neo-angiogenesis represents a potential strategy to treat cancer. Current anti-angiogenic therapies blocking tyrosine kinase receptors (TKR) such as VEGFR falter at the onset of resistance owing to upregulation of additional angiogenesis factors. Study and validation of these alternative neo-angiogenesis regulators as therapeutic targets is key, either alone or in combinatorial therapies. One promising candidate is apelin, the ligand for the G-protein-coupled APJ receptor and a potent angiogenic factor frequently upregulated in human cancer. Apelin is a secreted peptide specifically upregulated in cancer cells and tumour endothelial cells, which makes it an attractive target in cancer with potentially low toxicity. Using mouse models of cancer, we have now been able to prove for the first time that genetic targeting of Apelin significantly improves the survival of mice with Her2-positive mammary tumors (Figure 12). Importantly, genetic inactivation results in reduced numbers of blood vessels in NeuT-driven mammary tumors (Figure 13, left panel). Moreover, when we analysed *Apelin* mutant and repaired haploid ES cells for sprouting in our 3D blood vessel model, we found that VEGF-A induced blood vessel growth requires the expression of Apelin (Figure 13, right panel).

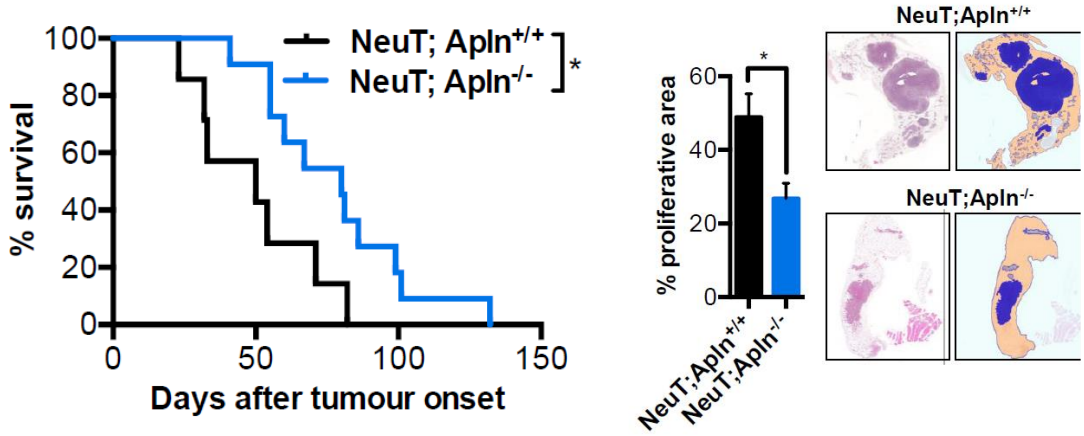

Figure 12. Targeting Apelin improves survival in mammary cancer and reduces tumour burden. Left panel, Kaplan Meier plot for survival after tumour onset in NeuT;Apln$^{+/+}$ (n=7) and NeuT;Apln$^{-/-}$ (n=11) mice with mammary cancer (P<0.02; Log rank test). Mice were sacrificed when the tumour size was 1cm^3, following the ethical guidelines. **Right panel**, Percentage of proliferative area in age-matched NeuT;Apln$^{+/+}$ (n=6) and NeuT;Apln$^{-/-}$ (n=8) mammary glands (P<0.05; T test). Representative images are shown. Left image: H&E staining of tumorigenic mammary gland. Right image: Proliferative tissue quantification using Definiens Tissue Studio TM followed by manual correction; normal mammary tissue is shown in orange and tumoural proliferative tissue in blue.

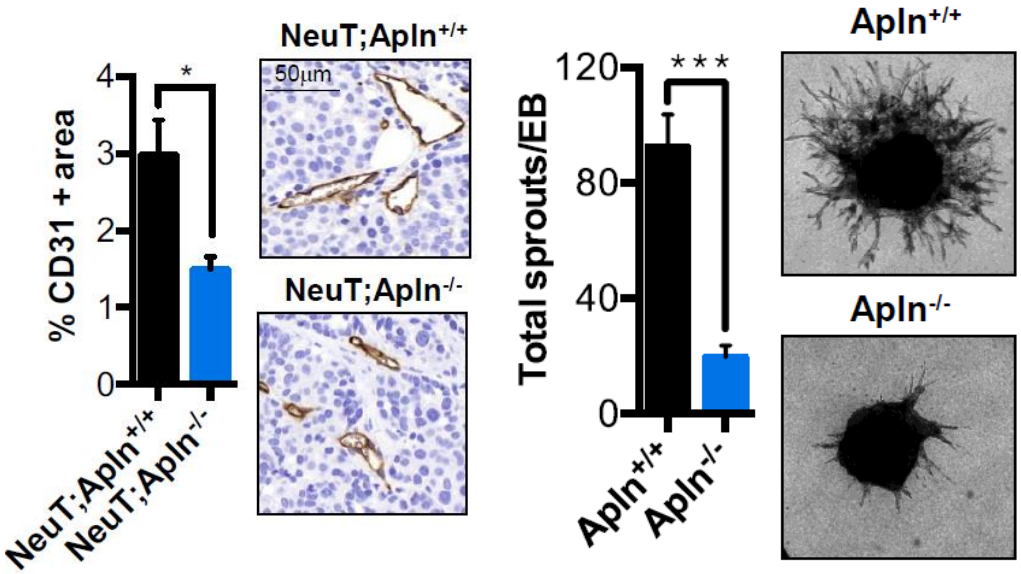

Figure 13. Targeting Apelin decreases tumour angiogenesis and disfavours VEGF-induced vessel sprouting. Left panel, Percentage of CD31-positive area in NeuT;Apln$^{+/+}$ (n=3) and NeuT;Apln$^{-/-}$ (n=3) age-matched mammary tumours (P<0.05; T test). **Right panel,** Total number of sprouts per Apln$^{+/+}$ (n=14) or Apln$^{-/-}$ (n=13) VEGF-stimulated embryoid body (EB) 5 days after initiation of the vessel sprouting (P<0.005; T test). Representative bright field images are shown.

Apelin-targeted tumors compared to wild-type controls not only present a decreased microvessel density but have less leaky vessels as revealed by over-time non-invasive MRI as well as quantification of dextran extravasation. Whereas current anti-angiogenic therapy (i.e. Sunitinib) presents a short vessel normalization window, we find that combining it with apelin ablation increases the normalization window to a greater extent than either approach alone. Moreover, we found that apelin and VEGF control key cell adhesion pathways in endothelial cells, which have been shown to be crucial in the regulation of vascular permeability. Accordingly, the combination of apelin-targeting and anti-VEGFR anti-angiogenic treatment significantly prolongs survival in mammary and lung cancer (Figure 14).

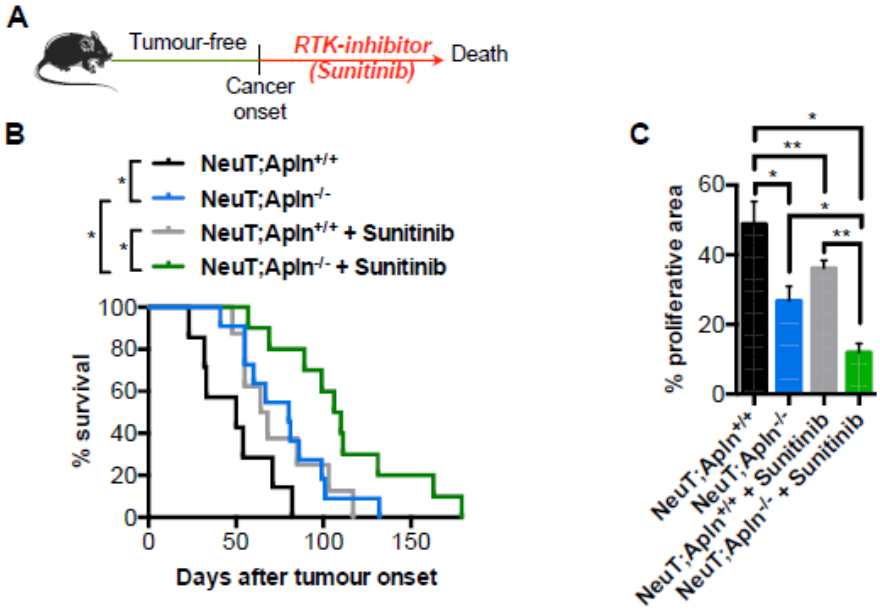

Figure 14. Apelin-targeting potentiates anti-angiogenic cancer therapy. A) Scheme of the experimental design followed. Anti-angiogenic treatment with Sunitinib Malate (60mg/kg per dose by gavage, three times a week) was started only after cancer onset, established by mammary tumour palpation and followed until the mice were sacrificed when the tumour size was 1cm^3, following the ethical guidelines. **B)** Kaplan Meier plot for survival in NeuT;Apln$^{+/+}$ and NeuT;Apln$^{-/-}$ mice with mammary cancer, control or treated with Sunitinib Malate after tumour onset (* indicates P<0.05; Log rank test; n=7-11). Mice were sacrificed when the tumour size was 1cm^3, following the ethical guidelines. **C)** Percentage of proliferative area in age-matched NeuT;Apln$^{+/+}$ and NeuT;Apln$^{-/-}$ mammary glands (P<0.05; T test) from control mice or mice treated with Sunitinib Malate (60mg/kg per dose) three times a week (n=3-7). Mammary glands were H&E stained and analyzed in a blinded and automated fashion,

Taken together, our data indicates that apelin is a potential novel target for cancer treatment. In combinatory strategies, apelin targeting could address the limitations of current anti-angiogenic therapy by prolonging tumor vessel normalization and, as a consequence, maintaining a diminished malignancy of the tumor for a longer period of time. This would not only increase the therapeutic window but it is also a common key feature to overcoming resistance to any tumor therapy.

RESEARCH ACCOMPLISHMENTS:

- **Generation of mice to assess the role of the RANKL/RANK system in BRCA1-driven mammary cancer**
- **Demonstration that RANK has a key role in BRCA1 mutant mammary cancer using two different genetic model systems**
- **RANK controls hormonal expansion of BRCA1/p53 mutant mammary stem cells**
- **Near genome wide fly screen to identify novel regulators of epithelial tumorigenesis and metastases.**
- **Cross-validation of our fly hits with thousands of human tumors and secondary validation of the top 100 tumor suppressor candidates**
- **Identification of TSPAN6 as a prognostic biomarker for a subtype of breast cancer**
- **Generation of two different TSPAN6 mutant mouse lines**
- **Experimental validation of TSPAN6 as a suppressor of hormone-induced breast cancer**
- **Development of efficient insertional mutagenesis vectors and set-up of near-genome wide screening system using haploid stem cells**
- **Generation of mutant ES cells clones with intragenic insertions covering more than 16000 mouse genes.**
- **Development of tissue engineered 3D blood vessel organoids from haploid ES cells**
- **Identification of Apelin as a key regulator of tumor neo-angiogenesis in breast cancer**

REPORTABLE OUTCOMES:

We are setting up a homepage (**Haplobank)** were we will deposit all our mutant ES cell clones. All insertions are mapped, linked to genome browsers, protein sites, PubMed, etc. All clones will be provided for cost and will be accessible to the research community. We hope that we become the key site for functional haploid ES cell genetics in the world providing technologies that should allow all cancer researchers in the world to rapidly perform experiments to validate cancer pathways, screen for pathways of toxic drugs, or elucidate synthetic lethals. The paper reporting the role of RANKL/RANK in BRCA1-driven breast cancer has been submitted. Moreover, the fly screen and work on TSPAN6 as well as the work on Apelin in angiogenesis are ready for submission.

CONCLUSIONS:

We combine fly genetics and near-genome wide mutagenesis using our haploid ES cells with the vision to perform rapid functional characterization of candidate breast cancer genes. Our transgenic RNAi library is covering the whole *Drosophila* genome, giving us an opportunity to examine the function of human candidate cancer genes. Using fly genetics, we have already discovered and experimentally validated a novel tumor suppressor gene, the surface receptor TSPAN6, in mammary cancer development. TSPAN6 expression also correlates with the prognosis of breast cancer in humans, providing a possible novel biomarker to stratify breast cancer risk. Moreover, we have made significant progress in mutating in essence every single mouse gene in a repairable fashion, using our haploid stem cell technologies. Our haploid ES cell library will be made available to all other breast cancer researchers. We have also managed to develop 3D blood vessel organoids form haploid ES cells using tissue engineering which allowed us to identify Apelin as an essential gene required for VEGFR induced blood vessel sprouting. Genetic ablation of Apelin indeed affects tumor neo-angiogenesis and improves survival in our NeuT-induced mammary cancer model. Moreover, Apelin appears to cooperate with VEGFR in blood vessel quality control and blood vessel leakiness in mammary cancers.

Finally, we have previously identified RANKL/RANK as a key "missing link" between sex hormones and the initiation of mammary cancer. We have now expanded this finding to cancer driven by BRCA1 mutations. Our results in mouse models indicate that genetic ablation of RANK markedly delays and in some cases even abolishes the development of BRCA1/p53 mutation-driven mammary cancer. Thus, if these data can be translated to humans, and considering that an anti-RANKL blocking antibody has already been approved for human use, our data provide a rationale for clinical trials to possibly prevent and delay the development of triple negative breast cancer.

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APPENDICES:

MPA/DMBA-induced mammary carcinogenesis. MPA/DMBA treatment was performed as described[3]. Briefly, six-week old female mice were anesthetized with ketamine-xylazine and surgically implanted with slow-release Medroxyprogesterone Acetat (MPA) pellets (50mg, 90-day release; Innovative Research of America) subcutaneously on the right flank. 200µl DMBA (5mg/ml diluted in cottonseed oil) was administered by oral gavage 6 times throughout the following 8 weeks. Onset of mammary tumors was determined by palpation.

Statistics. For the Kaplan-Meier analysis of tumor onset a log rank test was performed. $P < 0.05$ was accepted as statistically significant.

SUPPORTING DATA:

All relevant data are shown in the main description of the tasks.